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Cloned glutamic acid decarboxylase.

Isolated polypeptides useful in ameliorating autoimmune disease.

EP 0 519 469 A1

The present invention relates to the use of recombinant DNA technology for the transformation of a host organism with glutamic acid decarboxylase₆₅ (GAD₆₅) for the expression of GAD₆₅ polypeptides. Also encompassed are methods of using GAD₆₅ polypeptides diagnostically and therapeutically in autoimmune disease.

Insulin-dependent diabetes mellitus (IDDM; type I diabetes) is one of the most common metabolic disorders. In the United States, IDDM affects approximately one in 300 to 400 people, and epidemiological studies suggest that its incidence is increasing. The disease results from the autoimmune destruction of the insulin-producing β -cells of the pancreas. More specifically, the preonset stage is characterized by "Insulinitis", in which lymphocytes infiltrate the pancreatic islets and selectively destroy the β -cells. The typical IDDM presentation of hyperglycemia appears only after at least 80% of the insulin-producing β -cells are lost. The remaining β -cells are destroyed during the next few years.

Although insulin therapy allows most IDDM patients to lead normal lives, this replacement is imperfect and does not completely restore metabolic homeostasis. Thus, severe complications which result in dysfunctions of the eye, kidney, heart, and other organs are common in IDDM patients undergoing insulin therapy. Because of this, it is highly desirable to extend the latency period (e.g., through administration of immunosuppressant drugs) between the start of β -cell destruction and the actual requirement of insulin replacement (i.e., when 80% of the β -cells are destroyed). Therefore, a diagnostic test which determines the beginning of β -cell destruction would allow the clinician to administer immunosuppressant drugs (Silverstein, *et al.*, *New England Journal of Medicine*, 319:599-604, 1988) to extend this latency period and thus significantly delay the onset of insulin replacement side effects.

Many IDDM patients have sera which contain antibodies to a 64kD molecule (Baekkeskov, *et al.*, *J.Clin.Invest.*, 79:926-934, 1987; Atkinson, *et al.*, *Lancet*, 335:1357-1360, 1990), to islet cell cytoplasmic (ICA) molecules or islet cell surface (ICSA) molecules (Bottazzo, *et al.*, *Lancet*, 1:668-672, 1980), or to insulin (Palmer, *et al.*, *Science*, 222:1137-1139, 1983; Atkinson, *et al.*, *Diabetes*, 35:894-898, 1986). Atkinson and coworkers (Atkinson, *et al.*, *Lancet*, 335:1357-1360, 1990) have demonstrated that the presence of antibodies to the 64kD molecule in human sera appears to be the earliest and most reliable indicator that onset of IDDM symptoms will eventually occur.

Recently, Baekkeskov and coworkers established that the 64kD molecule and glutamic acid decarboxylase (GAD) have several antigenic epitopes in common and thus they may be identical or very similar molecules. Although this identification is an important finding, the use of this information as a diagnostic tool for predicting IDDM is quite cumbersome and limited unless knowledge of the molecular biology of GAD is known. Consequently, the cloning and subsequent production of large quantities of the 64kD molecule, or a GAD molecule which is antigenically substantially identical to the 64kD molecule, will allow the development of a diagnostic kit designed to predict IDDM. The present invention provides a means for accomplishing this result.

The present invention arose out of the discovery that recombinant DNA technology could be used to produce eukaryotic GAD₆₅ polypeptide and the GAD₆₅ polypeptide could be used in the diagnosis and therapy of patients with autoimmune disease. Particularly relevant is the use of cloned eukaryotic GAD₆₅ polypeptide in the diagnosis of patients having, or at risk of having, insulin-dependent diabetes mellitus (IDDM).

A major advantage of the present invention is that it provides the art with a ready source of eukaryotic GAD₆₅ polypeptide corresponding to that purified from natural sources, while avoiding the problems associated with the isolation of naturally occurring eukaryotic GAD₆₅ polypeptide when separating it from other eukaryotic non-GAD₆₅ polypeptides. This absence of other eukaryotic non-GAD₆₅ polypeptides is significant in that it allows the development of test systems which will only detect antibodies specifically reactive with GAD₆₅ polypeptides.

Another advantage of providing eukaryotic GAD₆₅ polypeptide in host cells is that by so doing, it is possible to obtain much larger quantities of the polypeptide than are currently practicably available from natural sources. As a consequence, not only is it possible to use the polypeptide of the invention to more accurately classify patients with such autoimmune diseases as IDDM, but it is also now possible to provide commercially useful quantities of GAD₆₅ polypeptide for use in diagnostic systems.

FIGURE 1 Cloning strategy for obtaining GAD₆₅ and GAD₆₇ specific cDNA probes.

FIGURE 2 DNA sequence and corresponding amino acid sequence for rat GAD₆₅.

FIGURE 3 DNA sequence and corresponding amino acid sequence for human GAD₆₅.

FIGURE 4 Comparison of rat GAD₆₅ and human GAD₆₅ amino acid sequences.

FIGURE 5 GAD₆₅ and GAD₆₇ cDNAs hybridize to different size RNAs.

FIGURE 6 Southern blots hybridized with cDNA probes specific for GAD₆₅ and GAD₆₇.

FIGURE 7 Immunological identification of GAD₆₅ and GAD₆₇.

The present invention relates to the manipulation of genetic materials by recombinant procedures which make possible the production of polypeptides possessing part or all of the primary structural conformation for one or more of the epitopes for binding autoantibodies to glutamic acid decarboxylase₆₅ (GAD₆₅). These polypeptides are highly useful for the immunological detection of autoantibodies reactive with them, since such autoantibodies are indicative of autoimmune diseases such as insulin dependent diabetes mellitus and "stiff man" syndrome. These polypeptides can also be used for purposes of screening drugs, such as those that alter GAD function, and for generation of polyclonal and monoclonal antibodies which, in turn, can be used diagnostically to detect GAD₆₅.

The development of specific DNA sequences encoding eukaryotic GAD₆₅ polypeptide for splicing into DNA vectors can be accomplished using a variety of techniques. For example, alternative methods which can be employed include (1) the isolation of a double stranded DNA sequence from the genomic DNA of the eukaryote; (2) the chemical manufacture of a DNA sequence to provide the necessary codons for the polypeptide of interest; and (3) the *in vitro* synthesis of a double stranded DNA sequence by reverse transcription of mRNA isolated from a eukaryotic donor cell. In the latter case, a double stranded DNA complement of mRNA is eventually formed which is generally referred to as cDNA.

The manufacture of DNA sequences is frequently the method of choice when the entire sequence of amino acid residues of the desired polypeptide product is known. When the entire sequence of amino acid residues of the desired polypeptide is not known, the direct manufacture of DNA sequences is not possible and the method of choice is the formation of cDNA sequences. Among the standard procedures for isolating cDNA sequences of interest is the formation of plasmid-carrying cDNA libraries which are derived from reverse transcription of mRNA which is abundant in donor cells that have a high level of genetic expression. When used in combination with polymerase chain reaction technology, even rare expression products can be cloned. In those cases where significant portions of the amino acid sequence of the polypeptide are known, the production of labeled single or double stranded DNA or RNA probe sequences duplicating a sequence putatively present in the target cDNA may be employed in DNA/DNA hybridization procedures which are carried out on cloned copies of the cDNA which have been denatured into a single stranded form (Jay, *et al.*, *Nucleic Acid Research*, 11:2325, 1983).

Hybridization procedures are useful for the screening of recombinant clones by using labeled mixed synthetic oligonucleotide probes wherein each is potentially the complete complement of a specific DNA sequence in the hybridization sample which includes a heterogeneous mixture of denatured double stranded DNA. For such screening, hybridization is preferably performed on either single stranded DNA or denatured double stranded DNA. These procedures are particularly useful in the detection of cDNA clones derived from sources where an extremely low amount of mRNA sequences relating to the polypeptide of interest are present. In other words, by using stringent hybridization conditions directed toward avoidance of non-specific binding, it is possible, for example, to allow the autoradiographic visualization of a specific cDNA clone by the hybridization of the target DNA to that single probe in the mixture which is its complete complement (Wallace, *et al.*, *Nucleic Acid Research*, 9:879, 1981).

In addition, a GAD cDNA library can be screened by injecting the various cDNAs into oocytes, allowing sufficient time for expression of the cDNA gene products to occur, and testing for the presence of the desired cDNA expression product, for example, by using antibody specific for GAD₆₅ polypeptide or by using functional assays for GAD₆₅ enzymatic activity.

Alternatively, a cDNA library can be screened indirectly for GAD₆₅ peptides having at least one epitope using antibodies to GAD₆₅ (Chang and Gottlieb, *J.Neurosci.*, 8:2123, 1988). Such antibodies can be either polyclonally or monoclonally derived and used to detect expression product indicative of the presence of GAD₆₅ cDNA. Preferred are antibodies directed to an epitope found in the first 100 amino acids of the N-terminal portion of GAD₆₅.

Of the three above-noted methods for developing specific DNA sequences for use in recombinant procedures, the use of genomic DNA isolates is the least common. This is especially true when it is desirable to obtain the microbial expression of mammalian polypeptides because of the presence of introns.

The present invention provides novel polypeptides of GAD₆₅ which have part or all of the primary structural conformation, that is, a continuous sequence of amino acid residues, having at least one epitope for antibodies to GAD₆₅.

It is possible to use the polypeptide fragments of the invention rather than intact GAD₆₅ to detect autoantibodies to GAD₆₅. The term "polypeptide," as applied to GAD₆₅ polypeptide, denotes any sequence of amino acids having an epitope for autoantibodies to GAD₆₅, wherein the sequence of amino acids is encoded by all or part of the cDNA sequences of the invention.

The polypeptides resulting from microbial expression of the DNA sequences of the invention can be further characterized by their freedom from association with other eukaryotic polypeptides or other

contaminants which might otherwise be associated with GAD₆₅ in its natural cellular environment or in such extracellular fluids as plasma or urine.

Studies by the present inventors unequivocally establish that GAD₆₅ and GAD₆₇ are encoded by distinct genes and are not produced, for example, by post-transcriptional or post-translational modification of a common genomic sequence. Evidence proving that GAD₆₅ and GAD₆₇ are encoded by different genes include: (a) the largest contiguous sequence of exact identity between GAD₆₅ and GAD₆₇ cDNAs is only 17 nucleotides in length, (b) cDNAs from GAD₆₅ and GAD₆₇ do not cross hybridize with each other's or with each other's mRNA under low stringency conditions (2.0 x SSC, 0.01% SDS, 23°C), and (c) GAD₆₅ and GAD₆₇ cDNAs do not cross hybridize with isolated genomic clones encoding GAD₆₇ and GAD₆₅, respectively.

The term "host" is meant to include not only prokaryotes, but also such eukaryotes as yeast, filamentous fungi, as well as plant and animal cells which can replicate and express an intron-free DNA sequence of eukaryotic GAD₆₅. However, prokaryotes are preferred as the host organism.

The term "prokaryotes" is meant to include all bacteria which can be transformed or transfected with the gene for the expression of GAD₆₅. Prokaryotic hosts may include gram negative as well as gram positive bacteria such as, for example, *E. coli*, *S. typhimurium*, *Serratia marcescens* and *Bacillus subtilis*.

A recombinant DNA molecule coding for the GAD₆₅ polypeptides can be used to transform or transfect the host using any of the techniques commonly known to those of ordinary skill in the art. Especially preferred is the use of a plasmid or a virus containing the GAD₆₅ coding sequence for purposes of prokaryotic transformation or transfection, respectively.

Methods for preparing fused, operably linked genes and expressing them in bacteria are well-known in the art (Maniatis, *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989). The genetic constructs and methods described therein can be utilized for expression of GAD₆₅ in prokaryotic hosts.

In general, expression vectors containing promotor sequences which facilitate the efficient transcription of the inserted eukaryotic genetic sequence are used in connection with the host. The expression vector typically contains an origin of replication, a promoter, and a terminator, as well as specific genes which are capable of providing phenotypic selection of the transformed cells. The transformed prokaryotic hosts can be grown in fermentors and cultured according to techniques known in the art to achieve optimal cell growth. The polypeptides of the invention can then be isolated from the grown medium, cellular lysates, or cellular membrane fractions.

The isolation and purification of the microbially expressed polypeptides of the invention may be by any conventional means such as, for example, preparative chromatographic separations and immunological separations such as those involving the use of monoclonal or polyclonal antibody.

By having provided the sequence of amino acid residues of GAD₆₅, the present invention provides for the manufacture of DNA sequences which code for the host expression of polypeptide analogs or derivatives of GAD₆₅ which differ from naturally-occurring forms in terms of the identity or location of one or more amino acid residues and which share some or all of the epitopes of naturally-occurring polypeptide forms.

The novel DNA sequences of the invention include all sequences useful in providing the expression in prokaryotic or eukaryotic host cells of polypeptides which have at least a part of the primary structural conformation for one or more epitopes capable of reacting with autoantibodies to GAD₆₅ which are comprehended by: (a) the DNA sequence as set forth in Figures 2 or 3 or their complementary strands (b) DNA sequences which hybridize to DNA sequences defined in (a) or fragments thereof; and (c) DNA sequences which, but for the degeneracy of the genetic code, would hybridize to DNA sequences defined in (a) and (b) above. Specifically comprehended in (b) are genomic DNA sequences which encode allelic variant forms of GAD₆₅. Part (c) specifically comprehends the manufacture of DNA sequences which encode GAD₆₅, GAD₆₅ fragments, and GAD₆₅ analogs wherein the DNA sequences thereof may incorporate codons which facilitate translation of mRNA in non-vertebrate hosts.

Since the cDNA sequence of the invention encodes essentially the entire human or rat GAD₆₅ molecule, it is now a matter of routine to prepare, subclone, and express smaller polypeptide fragments of cDNA from this or a corresponding cDNA sequence which would encode as few as one epitope for autoantibodies to human or rat GAD₆₅. The presence of such an epitope on a cloned polypeptide can then be confirmed using, for example, sera from a patient with autoantibodies to GAD₆₅. An example of such a smaller peptide is the first approximately 100 amino acids from the N-terminus of GAD₆₅ (shown in Figure 3). This amino acid sequence is essentially absent from GAD₆₇.

The GAD₆₅ of the invention is particularly suited for use in immunoassays in which it can be utilized in

liquid phase or bound to a solid phase carrier. In addition, GAD₆₅ used in these assays can be detectably labeled in various ways.

Examples of immunoassays which can utilize the GAD₆₅ of the invention are competitive and non-competitive immunoassays in either a direct or indirect format. Examples of such immunoassays are the radioimmunoassay (RIA), the sandwich (immunometric assay) and the Western blot assay. Detection of antibodies which bind to the GAD₆₅ of the invention can be done utilizing immunoassays which run in either the forward, reverse, or simultaneous modes, including immunohistochemical assays on physiological samples. The concentration of GAD₆₅ which is used will vary depending on the type of immunoassay and nature of the detectable label which is used. However, regardless of the type of immunoassay which is used, the concentration of GAD₆₅ utilized can be readily determined by one of ordinary skill in the art using routine experimentation.

The GAD₆₅ of the invention can be bound to many different carriers and used to detect the presence of antibody specifically reactive with the polypeptide. Examples of well-known carriers include glass, polystyrene, polyvinyl chloride, polypropylene, polyethylene, polycarbonate, dextran, nylon, amyloses, natural and modified celluloses, polyacrylamides, agaroses, and magnetite. The nature of the carrier can be either soluble or insoluble for purposes of the invention. Those skilled in the art will know of other suitable carriers for binding GAD₆₅, or will be able to ascertain such, using routine experimentation.

There are many different labels and methods of labeling known to those of ordinary skill in the art. Examples of the types of labels which can be used in the present invention include enzymes, radioisotopes, colloidal metals, fluorescent compounds, chemiluminescent compounds, and bioluminescent compounds.

Alternatively, the polypeptide of the invention can be used to detect antibodies to GAD₆₅ by measuring GAD enzymatic activity. For example GAD₆₅ and a specimen suspected of having antibodies to GAD₆₅ can be incubated for a period of time and under conditions sufficient to allow binding to occur between GAD₆₅ and the antibodies. The reaction product is precipitated and then tested for GAD enzymatic activity.

For purposes of the invention, the antibody which binds to GAD₆₅ of the invention may be present in various biological fluids and tissues. Any samples containing a detectable amount of antibodies to GAD₆₅ can be used. Normally, a sample is a liquid such as urine, saliva, cerebrospinal fluid, blood, serum and the like, or a solid or semi-solid such as tissue, feces and the like.

The materials for use in the assay of the invention are ideally suited for the preparation of a kit. Such a kit may comprise a carrier means being compartmentalized to receive in close confinement one or more container means such as vials, tubes and the like, each of the container means comprising one of the separate elements to be used in the method. For example, one of the container means may comprise GAD₆₅ bound to a carrier. A second container may comprise soluble, detectably-labeled second antibody, in lyophilized form or in solution.

In addition, the carrier means may also contain a plurality of containers each of which comprises different, predetermined amounts of GAD₆₅. These latter containers can then be used to prepare a standard curve into which can be interpolated the results obtained from the sample containing the unknown amount of autoantibodies to GAD₆₅.

In using the kit all the user has to do is add, to a container, a premeasured amount of a sample containing a measurable, yet unknown amount of autoantibodies to GAD₆₅ to be detected, a premeasured amount of carrier-bound GAD₆₅ present in the first container, and a premeasured amount of the detectably labeled second antibody present in the second container. Alternatively, the non-detectably labeled GAD₆₅ can be provided attached to the container to which the sample and the detectably labeled second antibody are added. After an appropriate time for incubation, an immune complex is formed and is separated from the supernatant fluid, and the immune complex or the supernatant fluid are detected, as by radioactive counting or addition of an enzyme substrate, and color development.

The term "ameliorate" denotes a lessening of the detrimental effect of the autoimmune response in the patient receiving therapy. The term "therapeutically effective" means that the amount of GAD₆₅ polypeptide used is of sufficient quantity to ameliorate the cause of disease due to the autoimmune response.

The recombinant GAD₆₅ polypeptides of the invention can also be used therapeutically in patients having an autoimmune response to GAD₆₅. Such therapy can be accomplished by, for example, the administration of recombinant GAD₆₅ polypeptide. Such administration can utilize unlabeled as well as labeled GAD₆₅ polypeptide. When unlabeled GAD₆₅ polypeptide is utilized advantageously, it would be in a form wherein, for example, the GAD₆₅ polypeptides are in fragments which are too small to stimulate an immune response, but large enough to bind, or block, the continuance of the autoimmune response. For example, GAD₆₅ could be digested enzymatically into epitope-sized peptides (typically 5-12 amino acids in length) and thereby bind to Fab binding portions present in the body fluids, or on the surface of immune cells, of the patient with autoimmune disease.

Alternatively, the recombinant GAD₆₅ polypeptides of the invention could be administered labeled with a therapeutic agent. These agents can be coupled either directly or indirectly to the GAD₆₅ polypeptides of the invention. One example of indirect coupling is by use of a spacer moiety. These spacer moieties, in turn, can be either insoluble or soluble (Diener, *et al.*, *Science*, 231:148, 1986) and can be selected to enable drug release from the GAD₆₅ polypeptide at the target site. Examples of therapeutic agents which can be coupled to the GAD₆₅ polypeptides of the invention for immunotherapy are drugs, radioisotopes, lectins, and toxins.

The drugs with which can be conjugated to the GAD₆₅ polypeptides of the invention include compounds which are classically referred to as drugs such as mitomycin C, daunorubicin, and vinblastine.

In using radioisotopically conjugated GAD₆₅ polypeptides of the invention for immunotherapy, certain isotopes may be more preferably than others depending on such factors as leukocyte distribution as well as stability and emission. Depending on the autoimmune response, some emitters may be preferably to others. In general, α and β particle-emitting radioisotopes are preferred in immunotherapy. Preferred are short range, high energy α emitters such as ²¹²Bi. Examples of radioisotopes which can be bound to the GAD₆₅ polypeptides of the invention for therapeutic purposes are ¹²⁵I, ¹³¹I, ⁹⁰Y, ⁶⁷Cu, ²¹²Bi, ²¹¹At, ²¹²Pb, ⁴⁷Sc, ¹⁰⁹Pd and ¹⁸⁸Re.

Lectins are proteins, usually isolated from plant material, which bind to specific sugar moieties. Many lectins are also able to agglutinate cells and stimulate lymphocytes. However, ricin is a toxic lectin which has been used immunotherapeutically. This is accomplished by binding the α -peptide chain of ricin, which is responsible for toxicity, to the antibody molecule to enable site specific delivery of the toxic effect.

Toxins are poisonous substances produced by plants, animals, or microorganisms that, in sufficient dose, are often lethal. Diphtheria toxin is a substance produced by *Corynebacterium diphtheria* which can be used therapeutically. This toxin consists of an α and β subunit which under proper conditions can be separated. The toxic A component can be found to GAD₆₅ polypeptide and used for site specific delivery to a leukocyte expressing a receptor for GAD₆₅ polypeptide.

Other therapeutic agents which can be coupled to the GAD₆₅ polypeptides of the invention, as well as *ex vivo* and *in vivo* therapeutic protocols, are known, or can be easily ascertained, by those of ordinary skill in the art.

The present invention also relates to a polypeptide which can be administered therapeutically to ameliorate the disease process in patients having, or at risk of having, this disease. The conventional single-letter code used to represent the various amino acids relates as follows:

Phe: F	Leu: L	Ile: I	Met: M
Val: V	Ser: S	Pro: P	Thr: T
Ala: A	Tyr: Y	His: H	Gln: Q
Asn: N	Lys: K	Asp: D	Glu: E
Cys: C	Trp: W	Arg: R	Gly: G

The polypeptide sequence of the invention was identified by comparing the amino acid sequences of human GAD₆₅, human GAD₆₇, and the P2-C protein of the picornavirus, coxsackie virus. The P2-C polynucleotide plays a role in the virus membrane bound replication complex. These analyses established the presence of an extensive sequence similarity between both GAD₆₅ molecules and the coxsackie virus. A core polypeptide of six contiguous amino acid residues of the GAD₆₅ and P2-C polypeptide are identical in amino acid sequence. Indeed, of the 24 amino acids in the polypeptide, 19 are identical or conserved. In addition, there also exists a high charge density and the presence of a proline residue which would render this region highly antigenic (see Table 1).

TABLE 1

<u>PROTEIN</u>	<u>AMINO ACID SEQUENCE</u>
Human GAD ₁₇	15 S I M A A R Y K Y F P E V K T K G M A A V P K L ₁₇
Human GAD ₁₅	15 A M M I A R F K M F P E V K E K G M A A L P R L ₁₅
10 Cocksackie Virus P2-C	9 F I E W L K V K I L P E V K E K H E F - L S R L ₉

In Table 1, the solid line encloses identical amino acids whereas the dashed line encloses amino acid residues with similar charge, polarity, or hydrophobicity.

The discovery of this common polypeptide region supports an etiologic role for "molecular mimicry" in the precipitation of diabetes. Thus, where a patient genetically susceptible to IDDM is infected by a coxsackie virus, the immune response to the coxsackie virus polypeptide results in a cross reactive immune response to the similar GAD sequence in the patient's β -cells. The immunological response is maintained by the antigenically similar GAD polypeptides resulting in the eventual destruction of the β -cells and the subsequent presentation of IDDM.

At present, it is believed that the elimination of pancreatic β -cells is mediated by a cellular autoimmune response. Consequently, a polypeptide of the invention should have the capability of blocking such cellular autoimmune response. Because of the complexity of autoimmune disease, it is possible to envision numerous possible therapeutic modalities which would allow the polypeptides of the invention to be used to ameliorate such diseases. Thus, it may be possible to utilize the polypeptides of the invention to block recognition by a specific T cell receptor (TCR) or an MHC receptor presenting an autoimmune antigen on the surface of an antigen presenting cell (APC). The inhibition of such recognition might occur, for example, by providing the patient with the polypeptide of the invention which, in turn, could displace the autoimmune antigen being presented in the antigen-cleft of the MHC receptor or, possibly, by direct interaction with the appropriate TCR on the surface of a T-helper cell. This latter therapeutic approach of direct interaction with the TCR could be achieved through induction of high-zone tolerance by use of high concentrations of soluble polypeptide.

Alternatively, the polypeptides of the invention could be used to stimulate a T-suppressor cell population in order to restore self-recognition and, thereby, ameliorate the autoimmune disease. Stimulation of T-suppressor cell populations could be achieved, for example, by use of a bi-specific antibody having one variable region specific for an epitope present on the autoimmune antigen residing in the cleft of the MHCII receptor and, a second variable region specific for an epitope present on the CD8⁺ receptor. The production of antibody specific for the polypeptide of the invention is a matter of routine to those of skill in the art, as is the preparation of bi-specific antibodies having specificity for 2 or more epitopes.

Polypeptide analogs of the present invention may be designed which will compete for recognition of self-antigens at the level of antigen presentation. Since MHC molecules contain a single peptide binding site, it is possible to design polypeptides which will bind with high affinity to disease-associated MHC molecules, but will not activate disease-causing T-helper cells. Such polypeptides act as antagonists for self-antigen recognition. Precedent for such an approach arises from observation that a mouse lysozyme polypeptide, itself non-immunogenic, can compete for MHC binding with an immunogenic polypeptide from hen-egg white lysozyme and thereby reduce T cell activation by that polypeptide (Adorini, *et al.*, *Nature*, 334:623-625, 1988). Similarly, such a therapeutic approach for screening effective polypeptide analogs has been utilized in such autoimmune diseases as experimental autoimmune encephalomyelitis (EAE) (Wraith, *et al.*, *Cell*, 59:248, 1989; Urban, *et al.*, *Cell*, 59:257, 1989).

The single-letter symbols used to represent the amino acid residues in the polypeptides of the present invention are those symbols commonly used in the art. The term "analog" refers to any polypeptide having a substantially identical amino acid sequence to a polypeptide provided herein and in which one or more amino acids have been substituted with chemically similar amino acids. For example, one polar amino acid, such as glycine or serine, may be substituted for another polar amino acid; or one acidic amino acid, such as aspartic acid may be substituted for another acidic amino acid such as glutamic acid; or a basic amino acid, such as lysine, arginine, or histidine may be substituted for another basic amino acid; or a non-polar amino acid such as alanine, leucine, or isoleucine may be substituted for another non-polar amino acid.

The term "analog" also means any polypeptide which has one or more amino acids deleted from or added to a polypeptide of the present invention, but which still retains a substantial amino acid sequence homology to such peptide. A substantial sequence homology is any homology greater than 50%. The term "fragment" means any shorter version of the polypeptides identified herein having at least 6 amino acid residues, wherein the fragment is capable of stimulating proliferation of islet infiltrating T lymphocytes (IITLs), or is a fragment capable of inhibiting the stimulation of such cells by a stimulating polypeptide fragment.

The term "chemical derivative" means any polypeptide derived from a polypeptide of the present invention and in which one or more amino acids have been chemically derivatized by reaction of the functional side groups of amino acid residues present in the polypeptide. Thus, a "chemical derivative" is a polypeptide that is derived from the sequences or polypeptides identified herein by one or more chemical steps. Such derivatized molecules include, for example, those molecules in which free amino groups have been derivatized to form amine hydrochlorides, P-toluene sulfoamides, benzoxycarboamides, T-butyloxycarboamides, thiourethane-type derivatives, trifluoroacetyl amides, chloroacetyl amides, or formamides. Free carboxyl groups may be derivatized to form salts, methyl and ethyl esters or other types of esters or hydrazides. Free hydroxyl groups may be derivatized to form O-acyl or O-alkyl derivatives. The imidazole nitrogen of histidine may be derivatized to form N-im-benzylhistidine. Also included as chemical derivatives are those polypeptides which contain one or more naturally occurring amino acids derivatives of the 20 standard amino acids. For example, 4-hydroxyproline may be substituted for proline; 5-hydroxylysine may be substituted for lysine; 3-methylhistidine may be substituted for histidine; homoserine may be substituted for serine and ornithine may be substituted for lysine.

It should be understood that the present invention is not limited to the illustrative polypeptides depicted in Table 1, instead, a polypeptide falling within the scope of this invention may extend outside of, or comprise less than, the region between amino acid 28 and amino acid 50 of coxsackie virus P2-C, or between amino acid 250 and amino acid 273 of GAD₆₅, or between amino acid 258 and amino acid 281 of GAD₆₇, as long as a substantial part of a given polypeptide is characterized by an amino acid sequence from that region, or segments or combinations thereof, and the polypeptide demonstrates the desired immunological or biological activity against autoimmune disease. In addition, polypeptides according to this invention include those having amino acid sequences which are longer or shorter in length than those of the polypeptides illustrated in Table 1, or which comprise segments or combinations thereof, as long as such polypeptides consist substantially of the region between the amino acids illustrated in Table 1 and demonstrate immunological or biological activity. Furthermore, polypeptides according to this invention include those characterized by a sequence of amino acids which is longer or shorter than that of the GAD₆₅ polypeptide of Table 1, or which comprise segments of that polypeptide and which display immunological or biological activity against the autoimmune disease. All polypeptides of the invention should not stimulate or enhance the autoimmune disease.

Accordingly, it should be understood that the specific selection of any one polypeptide within the polypeptides of the invention does not involve undue experimentation. Such a selection may be carried out by taking a number of polypeptides and testing them for their immunological and biological activity in ameliorating the autoimmune disease. The NOD mouse represents an excellent and well characterized model for screening polypeptides of the invention capable of ameliorating diabetes.

The polypeptides according to the present invention may be prepared by recombinant techniques or by conventional synthesis using known polypeptide synthetic methods, including synthesis on a solid support. An example of a suitable solid phase synthetic technique is that described by Merriweather (*J.Am.Chem.Soc.*, 85:2149, 1963). Other polypeptide synthetic techniques may be found, for example, in Bodanszky, *et al.*, *Peptide Synthesis*, John Wiley & Sons, 2d ed., 1976 as well as other references known to those skilled in the art. A summary of polypeptide synthesis techniques can be found in Stewart, *et al.*, *Solid Phase Peptide Synthesis*, Pierce Chemical Company, Inc., Rockford, Ill., 1984. The synthesis of polypeptides by solution methods may also be used, for example, as described in *The Proteins*, Vol. II, 3d ed., Neurath, *et al.*, eds., 105, Academic Press, New York, NY, 1976. Appropriate protective groups for use in such synthesis can be found in the above references as well as in J. McOmie, *Protective Groups in Organic Chemistry*, Plenum Press, New York, NY, 1973.

The polypeptides of the invention may also be prepared in an appropriate host transformed with DNA sequences that code for the desired polypeptide. For example, a polypeptide may be prepared by the fermentation of appropriate hosts that have been transformed with and which express a DNA sequence encoding the polypeptide. Alternatively, a DNA sequence encoding several of the polypeptides of this invention may be linked together and those sequences may then be used to transform an appropriate host to permit the expression of polypeptides involved in the autoimmune disease.

The dosage ranges for the administration of the GAD₆₅ polypeptides of the invention are those large enough to produce the desired effect in which the symptoms or cellular destruction of the autoimmune response are ameliorated. The dosage should not be so large as to cause adverse side effects, such as unwanted cross-reactions, anaphylactic reactions, and the like. Generally, the dosage will vary with the age, condition, sex, and extent of the disease in the patient and can be determined by one of skill in the art. The dosage can be adjusted by the individual physician in the event of any counterindications. Dosage can vary from about 0.1mg/m² to about 2000mg/m², preferably about 0.1mg/m² to about 500mg/m²/dose, in one or more dose administrations daily, for one or several days.

The GAD₆₅ polypeptides of the invention can be administered parenterally by injection or by gradual perfusion over time. The GAD₆₅ polypeptides of the invention can be administered intravenously, intraperitoneally, intra-muscularly, subcutaneously, intracavity, or transdermally.

Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solution, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils, intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, anti-oxidants, chelating agents, and inert gases and the like.

The invention also relates to a method for preparing a medicament or pharmaceutical composition comprising the GAD₆₅ polypeptides of the invention, the medicament being used for therapy of autoimmune response to GAD₆₅.

The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific examples which are provided herein for purposes of illustration only and are not intended to limit the scope of the invention.

EXAMPLE 1

CLONING AND EXPRESSION OF GAD₆₅

A. RECOMBINANT DNA PROCEDURES

In order to obtain cDNA probes specific for GAD₆₅ and GAD₆₇, total RNA was extracted from adult rat brain by guanidine isothiocyanate-casium gradient using the method of Chirgwin, *et al.* (*Biochemistry*, 18:5294, 1979). Poly (A) RNA was purified on oligo dT cellulose, using the protocol by Bethesda Research Laboratories (BRL). First strand synthesis was performed by using MMLV-reverse transcriptase (BRL), with conditions suggested, except that poly d(N₆)-mers (Pharmacia) were used as primers. This cDNA-RNA mixture was heat inactivated at 65°C for 15 min and stored at -20°C. For PCR, 1/50 of the sample was added to the 100μl reaction. Degenerate oligonucleotides were synthesized (Applied Biosystems) to encode the underlined common amino acid sequences of feline (from cDNA) (Kobayashi, *et al.*, *J.Neurosci.*, 7:2768, 1987) and rat (from peptides) (Chang and Gottlieb, *J.Neurosci.*, 8:2123, 1988) GAD (Figure 1). The 5'-end sequence of each degenerate oligonucleotide contained one strand of the DNA sequence recognized by either SstI and HindIII (5' oligo) or SstI and SstII (3'-end oligo). These primers were used for selective amplification by polymerase chain reaction of the generated cDNA template as described by Gould, *et al.* *Proc.Natl.Acad.Sci.,USA.* 86:1934, 1989). PCR products were subcloned into HindIII/SstI double digested Bluescript SK vector (Stratagene), transformed into DH5 (BRL), and plated by standard methods (Maniatis, *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989).

Colony hybridization was done with an 5'-³²P end labeled oligonucleotide specific to feline GAD₆₇ - (Kobayashi, *et al.*, *J.Neurosci.*, 7:2768, 1987). End labeling of oligonucleotide, hybridization conditions, and washing conditions were done as described (Wallace, *et al.*, in *Guide to Molecular Cloning Techniques*; Berger, *et al.*, Eds. in *Methods of Enzymology*; Abelson, *et al.*, Eds. Academic Press, Inc., San Diego, 432-442, 1987), except that the nitrocellulose filters were washed at 50°C for 15 min. Colonies which were positive and negative in the hybridization were individually picked and grown overnight in Terrific Broth (Tartof, *et al.*, *Focus*, 9:12, 1987). DNA was isolated using a boiling method (Maniatis, *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989) and templates were denatured by 0.2N NaOH and purified by Sephacryl S400 spun columns (Pharmacia). Sequencing of denatured double stranded template was by the chain-termination method (Sanger, *et al.*,

Proc.Natl.Acad.Sci.,USA, 74:5463. 1977) using the T7-sequencing kit (Pharmacia).

As shown in Figure 1, PCR-generated rat GAD₆₅ and GAD₆₇ cDNAs were used as probes to screen a lambda ZAP (Stratagene) rat hippocampus library provided by S Heinemann (Salk Institute) by standard techniques (Maniatis, *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989) A 2400 nucleotide GAD₆₅ cDNA (the largest clone) was isolated and subcloned by "zapping" as described by Stratagene. When a rat GAD₆₇ cDNA was obtained which was smaller than a 3.2kb rat GAD₆₇ cDNA clone already on hand, the larger cDNA was sequenced. Exo III deletions (Henikoff, *Gene*, 28:351, 1984) were made in both directions for GAD₆₅ and GAD₆₇ and templates were prepared and sequenced as described above. Anchored PCR (Frohman, *et al.*, *Proc.Natl.Acad.Sci.,USA* 85:8998, 1988) was done to done the remaining 5'-ends of GAD₆₅ and GAD₆₇ mRNAs which were not represented in the original cDNA clones isolated in the library screening. Sequencing of these clones revealed that neither GAD₆₅ nor GAD₆₇ mRNAs contained any further initiation codons (AUGs) in frame with the previously designated initiation codons of the original cDNA clones.

EXAMPLE 2

CHARACTERIZATION OF CLONED GAD₆₅

A. NORTHERN BLOT HYBRIDIZATION

Two PCR-derived cDNA probes were hybridized to Northern blots containing rat brain RNA in order to determine whether the GAD₆₇ and GAD₆₅ cDNAs were derived from two different mRNAs. RNA was extracted as described in Example 1. Poly (A) RNA was separated by electrophoresis in formaldehyde and transferred onto Biotrans (ICN) membranes, and hybridization was performed as described by Well, *et al.* (*J.Neurosci.*, 16:311, 1986), except that 100µl/ml of poly (A) was added. Probes were labeled to approximately 10⁹ dpm/µg by the oligolabeling procedure of Feinberg and Vogelstein (*Anal.Biochem.*, 132:6, 1983). Identical results were subsequently obtained with full-length clones of GAD₆₅ and GAD₆₇ cDNAs.

As shown in Figure 5, lanes 1 and 2 contain 1µg of poly (A) selected RNA extracted from rat cerebellum. Lane 1 was hybridized to a cDNA probe for the rat cognate of feline GAD₆₇ (Kobayashi, *et al.*, *J.Neurosci.*, 7:2768, 1987) and lane 2 with a cDNA probe for the rat peptide sequence (which corresponds to GAD₆₅).

The cDNA probe for the rat peptide sequence hybridized to a 5.7kb RNA, while the cDNA probe for the rat cognate of our feline cDNA, hybridized to a 3.7kb RNA. This demonstrated that GAD₆₅ and GAD₆₇ are not derived from the same mRNA.

B. GENOMIC HYBRIDIZATION OF GAD₆₇ AND GAD₆₅

In order to investigate the possibility that GAD₆₇ and GAD₆₅ arise from separate genes, cDNAs of both GAD₆₇ and GAD₆₅ were hybridized to DNA blots containing genomic DNA.

For Southern blots, genomic DNA was extracted from rat liver as described (Kaiser, *et al.*, in *DNA Cloning*, vol.I, A Practical Approach, D.M. Glover ed., IRL Press, Oxford, 38-40, 1985). DNA (10µg/sample) was digested to completion with EcoRI and HindIII using conditions recommended by the suppliers (BRL, Gaithersburg, MD). DNA fragments were separated by electrophoresis at 1.5v/cm for 16 hrs in 0.8% agarose. The DNA was then transferred to Zeta-Probe membranes (Bio-Rad), hybridized, and washed, as described by Gatti, *et al.* (*Biotechniques*, 2:148, 1984), except that 5µg/ml Carnation dried milk was substituted for Denhardt's solution. Probes for Southern blots were labeled as described in Example 1, above.

As shown in Figure 6, genomic DNA digested with HindIII and EcoRI are in lanes 1 and 3 and lanes 2 and 4, respectively. GAD₆₇ cDNA was hybridized to lanes 1 and 2, whereas GAD₆₅ cDNA was hybridized to lanes 3 and 4. Numbers along the side of the gel are the DNA fragment sizes in kilobases.

This data shows that the two cDNAs hybridize to genomic fragments of different sizes. In addition, the greatest contiguous stretch of identical nucleotide sequence of GAD₆₅ and GAD₆₇ cDNAs is only 17 nucleotide bases in length. Thus, GAD₆₇ and GAD₆₅ are encoded by two distinct genes.

C. ENZYMATIC COMPARISON OF GAD₆₇ AND GAD₆₅

Studies were done comparing the effect of PLP on the activity of GAD₆₇ and GAD₆₅. In so doing, both cDNAs were subcloned into vectors that allowed their expression in bacteria (Studier, *et al.*, *J.Mol.Biol.*,

189:113, 1986). Overexpression of "fusionless" GAD₆₅ and GAD₆₇ was accomplished by subcloning GAD₆₅ cDNA into the NcoI site of pET-8c and GAD₆₇ cDNA into the NheI site of pET-5c vectors (Studier, *et al.*, *J.Mol.Biol.*, 189:113, 1986).

To obtain compatible sticky ends for correct in-frame subcloning of both cDNAs, selective amplification was performed by PCR using conditions suggested by United States Biochemical (USB), with 200μM dNTPs and 1.5mM MgCl₂ in the mixture and annealing at 55°C with 20 cycles to decrease infidelity of AmpliTaq (USB) Primers specific for GAD₆₅ and GAD₆₇ contained one DNA strand of the NcoI and SpeI recognition sites, respectively. Since there is a NheI restriction site within the coding region of GAD₆₇, SpeI (which is compatible with NheI) was used.

PCR products were subcloned into their respective pET vectors, transformed into DH5 and plated as described (Maniatis, *et al.*, *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989). Colonies were picked and grown overnight in LB broth with 50μg/ml ampicillin. Subclones with correct orientation were transformed into BL21(DE3) strain (Studier, *et al.*, *J.Mol.Biol.*, 189:113, 1986) for overexpression. As a negative control, the pET-8C vector with no insert was transformed and subsequently induced. Single colonies were picked, grown, induced by 1mM isopropyl-B-D-thiogalactopyranoside (IPTG), and analyzed on SDS-PAGE gels as described (Sambrook, *et al.*, *Molecular Cloning a Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor 17.15-17.16, 1989).

To measure GAD activity, we induced 10ml cultures of bacteria at OD₆₀₀-0.5 with 1mM IPTG. Two hours after induction, bacteria was spun down and resuspended and sonicated in 1ml of homogenizing buffer (1mM phenylmethylsulfonyl fluoride (PMSF), 1mM 2-aminoethylisothiuronium bromide (AET), and 60mM potassium phosphate, pH 7.1) After sonication, cell debris was removed by centrifugation and protein concentration was measured (Bradford, *Anal.Biochem.*, 72:248, 1986) in the supernatant (supernatant was stored in aliquots at -70°C). Brain homogenates were prepared as described (Legay, *et al.*, *J.Neurochem.*, 46:1478, 1986). GAD activity was measured as described (Krieger, *et al.*, *J.Neurochem.*, 33:299, 1984) with 0.2mM PLP present or absent and 20μl of brain homogenate or bacterial lysate in the incubation mixture. Production of ¹⁴CO₂ in bacterial lysates was linear with respect to time of incubation and protein concentration.

TABLE 2

Source	GAD Specific Activity ^a		Fold Increase in Induction
	- PLP	+ PLP	
BL21(DE3) + pET-8c	12 ± 0.4	9 ± 1	—
BL21(DE3) + pET-GAD ₆₅	115 ± 3	773 ± 61	6.7
BL21(DE3) + pET-GAD ₆₇	160 ± 2	389 ± 8	2.4
Rat Brain	131 ± 5	216 ± 2	1.6

^a cpm of ¹⁴CO₂/μg protein/hr of triplicates ± S.E.M.

As shown in Table 2, bacterial lysates containing GAD₆₅ or GAD₆₇ catalyze the conversion of [1-¹⁴C]-glutamate to GABA and ¹⁴CO₂.

PLP stimulates the enzymatic activity of GAD₆₅ more than GAD₆₇. This greater stimulation probably reflects the faster cycling of GAD₆₅ through the inactivation cycle proposed by Martin and coworkers (Martin, *Cell.Mol.Neurobiol.*, 7:237, 1987). This faster cycling suggests that GAD₆₅ contributes more to the pool of apo-GAD that exists *in vivo* (Miller, *et al.*, *Brain Res.Bull.*, 5(Suppl.2):89, 1980). Thus, *in vivo*, PLP appears to regulate GAD₆₅ activity more than GAD₆₇ activity.

GAD₆₅ activity in bacterial lysates is similar to the five-fold PLP stimulation of GAD activity found in synaptosomes prepared from rat substantia nigra (Miller, *et al.*, *J.Neurochem.*, 33:533, 1979). Because both GADs are more dependent upon added PLP in bacteria than is the GAD activity in crude rat brain homogenates, the endogenous PLP concentration of bacterial lysates may be less than rat brain homogenates.

D. IMMUNOLOGICAL IDENTIFICATION OF GAD₆₅ AND GAD₆₇

Rat brain homogenates and bacterial lysates were extracted as described above. Equal volumes of loading buffer were added to each sample as described (Harlow, *et al.*, *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988) Proteins were separated by electrophoresis

in a 10% acrylamide gel in SDS and electrophoretically transferred to nitrocellulose (Harlow, *et al.*, *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 1988). The unreacted sites were blocked with a phosphate buffered saline (PBS) solution containing 2% bovine serum albumin (fraction V), 1% gelatin, and 1% Triton-X-100 at 42°C for one hr. After washing, the nitrocellulose filter was then cut into three sections and incubated with the following primary antibodies: lanes 1 to 4 with a 1/2000 dilution of the antiserum of Oertel, *et al.* (*Neuroscience*, 6:2689, 1981), which recognizes both GAD₆₇ and GAD₆₅; lanes 5-8 with a 1/2000 dilution of K-2 antiserum, which recognizes only GAD₆₇; lanes 9-12 with a 1/2000 dilution of GAD-6 monoclonal antibody, which is specific for GAD₆₅ (Chang, *et al.*, *J.Neurosci.*, 8:2123, 1988). All filters were extensively washed and appropriate secondary antibodies were incubated and washed. Bound antibodies were detected with ¹²⁵I-labeled protein A and autoradiography. Each lane contained the following: lanes 1, 5, and 9 are BL21(DE3) + pET-GAD₆₇; lanes 2, 6, and 10 are BL21(DE3) + pET-GAD₆₅; lanes 3, 7, and 11 are rat brain homogenate; and lanes 4, 8, and 12 are BL21-(DE3) + pET-8c.

The immunoblots of bacterially produced GAD₆₅ and GAD₆₇ demonstrated that GAD₆₅ indeed corresponds to the smaller GAD in brain extracts, and GAD₆₇ to the larger form (Figure 7). Previous work has demonstrated the correspondence of GAD₆₇ to the larger GAD for feline GAD₆₇, and for mouse GAD₆₇ - (Katarova, *et al.*, *Eur.J.Neurosci.*, 2:190, 1990; 235, 1987). The mobilities of bacterially produced GAD₆₅ and GAD₆₇ (as detected with the antiserum of Oertel, *et al.*, (*Neuroscience*, 6:2689, 1981) are identical to the immunoreactive doublet seen in rat brain homogenate.

The smaller molecular weight and larger molecular weight forms of GAD in rat brain are thus identical in antigenicity and size to the products of GAD₆₅ and GAD₆₇ cDNAs, respectively. Consequently, the two GADs in rat brain are GAD₆₅ and GAD₆₇. From this data it can also be concluded that the molecular identity of the reported PLP-dependent and PLP-independent GADs by Tapia (Bayon, *et al.*, *J.Neurochem.*, 29:519, 1977) are GAD₆₅ and GAD₆₇, respectively. Martin and coworkers (Spink, *et al.*, *Brain Res.*, 421:235, 1987) have reported the existence of four kinetically different forms of rat brain GAD. However, immunoblotting experiments (with the antisera used here) of these forms have not been reported.

E. DISTRIBUTION OF GAD₆₅ AND GAD₆₇ IN RNAs BRAIN TISSUE

Experiments were done to determine the distribution of GAD₆₅ and GAD₆₇ in RNAs in cerebellum using *in situ* hybridization.

Transcripts of, respectively, 3.2kb and 2.3kb from GAD₆₅ and GAD₆₇ cDNAs, were radiolabeled with ³⁵S according to Wuenschell, *et al.* (*Proc.Natl.Acad.Sci.,USA*, 83:6193, 1986) procedure. Hydrolyzed fragments of 200 bp were hybridized to coronal sections of a rat cerebellum. Animals were anesthetized under halothane and decapitated. The brain was rapidly frozen in dry ice and coronal frozen sections (12μm) were fixed for 30 min in freshly prepared 4% formaldehyde in phosphate-buffered saline (PBS; 130mM NaCl, 10mM Na phosphate, pH 7.0). The tissue was dehydrated through graded ethanol solutions and stored at -70°C.

In order to increase tissue permeability, the sections were submitted to the following pretreatments, rehydration through graded ethanol solutions (5 min each in 95%, 85%, 70%, 50%, and 30% ethanol); PBS (5 min); 0.02N HCl (10 min); PBS (5 min); 0.01% Triton N-101 in PBS (1 min); PBS (2 x 5 min); 1 μg/ml proteinase K (7.5 min); and glycine to inhibit proteinase K in PBS (3 x 5 min). Proteinase K was digested for 30 min at 37°C before use. Sections were then incubated at 37°C in 50% formamide, 750mM NaCl, 25mM EDTA, 0.2% SDS, 0.02% BSA, 0.002% Ficoll, 0.02% polyvinylpyrrolidone, 250μg/ml yeast tRNA, 250μg/ml poly A, and 25mM PPES (pH 6.8).

For the hybridization, 100mM DTT, 10% dextran sulfate, and sense or antisense ³⁵S-RNA were added to the prehybridization solution. An aliquot (50μl) of the hybridization solution containing about 3 ng (10⁶ cpm) of probe (sense or antisense) was added onto the slides. Each slide was coverslipped and incubated for 16 hrs at 50°C, following which the siliconized coverslips were removed by brief washing in 4 x SSC (1 x SSC - 150mM NaCl, 60mM Na citrate, pH 7.0).

Sections were then treated with ribonuclease A (50μg/ml in 0.5M NaCl, 10mM Na thiosulfate, 1mM EDTA, 10mM TrisHCl pH 8.0) for 20 min at 37°C and rinsed for 2 hrs at room temperature in 2 x SSC, 10mM Na thiosulfate, for 30 min at 55°C. Sections were dehydrated in ethanol, delipidated in xylene, coated with Kodak NTB2 emulsion and exposed for 10 days at 4°C. The emulsion was developed with Kodak D19, and the tissue counterstained with cresyl violet.

Autoradiographic grains were detected using reflected polarized light and grain numbers, densities, and cell areas were determined with an Analytic Imaging Concepts image analyzer system. Due to the low background level, the criteria for defining a cell "labeled" was based on the presence of more than 5

clustered grains. The GAD labeled cells were found scattered throughout the brain, enabling the measurement of the number of grains over individual cells. The boundary of the cell and the area covered by a grain allowed the calculation of the number of grains per cell. This analysis was done at a high magnification (800X), using both reflected polarized light and transmitted light to simultaneously visualize the stained cell and the superimposed grains. Numbers are means \pm S.E.M. of "n" cells.

TABLE 3

CELL TYPE	GRAINS/CELL		GAD ₆₇ :GAD ₆₅
	GAD ₆₇ mRNA	GAD ₆₅ mRNA	
Purkinje	172 \pm 34 (87) ^a	43 \pm 2 (70)	4.0
Golgi II	96 \pm 8 (80)	64 \pm 9 (65)	1.5
Basket	61 \pm 12 (102)	16 \pm 1 (57)	3.8
Stellate	55 \pm 15 (65)	18 \pm 3 (37)	3.1

^a \pm S.E.M.(n)

In all neuronal types GAD₆₇ mRNA levels are greater. The observations with *in-situ* hybridization are consistent with previous findings (Nitsch, *J.Neurochem.*, 34:822, 1980; Denner, *et al.*, *J.Neurochem.*, 44:957, 1985; Itoh, *et al.*, *Neurochem. Res.* 6:1283, 1981) that the ratio of PLP dependent to independent GAD activities in the cerebellum is one of the lowest in brain regions tested. In addition, as shown in Table 3, the order of amounts for GAD₆₇ mRNA is Purkinje > Golgi II > Basket > Stellate cells; in contrast, for GAD₆₅ mRNA, this order is Golgi II > Purkinje > Basket > Stellate cells.

The expression of GAD₆₅ and GAD₆₇ mRNAs thus differs among classes of neurons. The contribution of each to total GAD activity in turn affects how GABA production is regulated. For example, the substantia nigra contains one of the highest ratios of PLP-dependent to PLP-independent GAD activities (Nitsch, *J.Neurochem.*, 34:822, 1980), increasing GABA concentration in the substantia nigra by local injection of inhibitors of GABA catabolism is especially effective in reducing seizure susceptibility (Gale, *Fred. Proc.*, 44:2414, 1985). Experimental animals undergoing seizures induced by PLP-antagonists may therefore be unable to inhibit seizure propagation because of inhibition of GAD₆₅ particularly in nerve terminals within the substantia nigra.

F. SUBCELLULAR LOCATION OF GAD₆₅ AND GAD₆₇

The distribution of GAD₆₅ and GAD₆₇ was evaluated in the S₂ and synaptosome subcellular fractions. S₂ is a high speed supernatant consisting of the cytosol of all cells in the brain, while the synaptosomal fraction consists primarily of nerve endings (Gray, *et al.*, *J. Anat., Lond.*, 96:79, 1962). For these studies, whole rat brain fractionation was performed as described by Booth and Clark (Booth, *et al.*, *Biochem. J.*, 176:365, 1978). Protein concentrations were determined by Schaffner and Weissman (Schaffner, *et al.*, *Anal. Biochem.* 56:502, 1973). Samples were prepared as described (Kaiser, *et al.*, *DNA Cloning, Vol. I, A Practical Approach*, D.M. Glover ed. (IRL Press, Oxford, 1985, pp. 38-40), and immunoblotting was done as described above using GAD-6 monoclonal antibody and K-2 antiserum. Equal amounts of protein (16 μ g) were added to each lane. Autoradiography showed a linear response of increasing amount of ¹²⁵I-protein A bound to antibody with protein concentrations of 1, 3, 10, 30, 100 μ g with both K-2 antiserum and GAD-6 monoclonal antibody (data not shown).

The results showed that GAD₆₇ was present in equal amounts in both fractions. Since the S₂ fraction contains the cytosolic proteins of glial (as well as other non-neuronal) and neuronal cells, the concentration of GAD₆₇ must be greater in neuronal cell bodies than in nerve endings. In contrast, the concentration of GAD₆₅ was greater in synaptosomes than in S₂. These subcellular fractionation experiments suggest that in contrast to GAD₆₅, a much greater fraction of GAD₆₇ is present in cell bodies of neurons than in nerve terminals. Thus, subcellular fractionation, like immunohistochemistry, shows that GAD₆₅ and GAD₆₇ have different subcellular distributions.

In vivo experiments utilizing inhibitors of GABA synthesis and degradation have suggested that the GABA pool in neuronal cell bodies is different from that in the nerve terminals (Iadarola, *et al.*, *Mol. Cell. Biochem.*, 39:305, 1981). GABA produced by GAD₆₇ may be involved more in cellular metabolism (for example, in the GABA shunt) and in dendrodendritic synapses. The dendrites of granule cells in the olfactory bulb, which form dendrodendritic synapses with mitral dendrites (Shepard, *Physiol. Rev.*, 52:864,

1972) and probably release GABA (McLennan, *Brain Res.*, 29:177-184, 1971), label intensely with K-2 antiserum. While not shown here, it has also been found greater GAD₆₇ than GAD₆₅ mRNA levels (2-3 fold) in the olfactory bulb. This distribution is consistent with the reported finding that most GAD activity in the olfactory bulb is present in S₂ and P₁ (crude nuclear pellet) and not in synaptosomes (Quinn, *et al.*, *J. Neurochem.*, 35:583, 1980).

The differing subcellular distributions of GAD₆₅ and GAD₆₇ could result from cytoskeletal anchoring or from some unknown protein targeting mechanism. Some cytoskeletal proteins have distributions that resemble GAD₆₅ and GAD₆₇. For example, in cultured sympathetic neurons Peng, *et al.* (*J Cell. Biol.*, 102:252, 1986), demonstrate that 84% of tau is in axons while 100% of MAP-2 is in cell bodies and dendrites. In addition, 43kd protein, a cytoskeletal protein, is thought to anchor the acetylcholine receptor to the underlying membrane cytoskeleton (Flucher, *et al.*, *Neuron*, 3:163, 1989).

EXAMPLE 3

DETECTION OF GAD AUTOANTIBODIES IN CLINICAL SPECIMENS

A. MATERIALS AND METHODS

1. Patient Specimens. Sera from four groups of individuals were selected from a previous study by Atkinson and co-workers (Atkinson, *et al.*, *Lancet*, 335:1357-1360, 1990). These groups consisted of: Group (1), 1 new onset IDD patients diagnosed according to the established National Diabetes Data Group (NDDG) criteria (Gleichman, *et al.*, *Diabetes*, 36:578-584, 1987) that had been referred to the University of Florida, Diabetes Clinics; Group (2), 5 randomly selected islet cell cytoplasmic antibody (ICA) negative non-diabetic controls without any known family history of autoimmune disease; Group (3), 13 individuals whose sera had been collected 3 to 66 months prior to their documented clinical onsets of IDD; Group (4), non-diabetic controls and relatives, and those who were studied prior to their onsets of IDD; and Group (5), 3 patients at risk for IDDM, but where onset has not yet occurred. This latter group had been ascertained through ongoing prospective ICA screening studies of more than 5000 first degree relative of IDD probands, and 8200 individuals from the general population (of which 4813 were school children).

2. Islet Cell Autoantibodies. ICA were assayed by indirect immunofluorescence on blood group O cryocut pancreatic (Atkinson, *et al.*, *Lancet*, 335:1357-1360, 1990). All results were interpreted on coded samples, with control negative and positive sera in each batch. The degrees of ICA positivity were analyzed with the guidelines established by the Immunology Diabetes Workshop (IDW) for the standardization of ICA (Gleichman, *et al.*, *Diabetes*, 36:578-584, 1987). All positive sera were titrated by end point dilution, and the Juvenile Diabetes Foundation (JDF) units were determined by reference to a standard serum previously calibrated to the international JDF standard of 80 units. In this studies reported here, a positive ICA result was defined by replicate titers of 10 JDF units or greater.

3. HLA DR Typing. HLA DR typing was performed as adapted from the method described by Van Rood and Van Leuwen (*Nature*, 262:795-797, 1976), using DR trays (One Lambda Laboratories, Los Angeles, CA).

4. Human Islet Cells. Human pancreatic islets were isolated from cadaveric pancreases and maintained *in vitro* as previously described (Ricordi, *et al.*, *Diabetes*, 37:413-420, 1988). The islet cells were metabolically labeled with ³⁵S methionine (Amersham, Arlington Heights, IL) *in vitro* (95% air/5%CO₂).

5. Islet Cell Extractions and Immunoprecipitations. Islet cells were extracted as previously described by Atkinson, *et al.* (*Lancet*, 335:1357-1360, 1990) with the following modifications. For immunoprecipitation studies, the islet cell lysates were precleared twice by incubation (2h, 4°C) with either control, IDD serum (100μl), or GAD-6 (Chang, *et al.*, *J.Neuro*, 8:2123-2130, 1988) (1μl in 99μl of Tris buffer (Atkinson, *et al.*, *Lancet*, 335:1357-1360, 1990) for every 1000 islets. Immune complexes were then absorbed (1h 4°C) with an excess of protein A Sepharose CL-4B (Pharmacia, NJ). Aliquot volumes representing 1000 islet cells containing unbound (precleaned) lysate were then incubated (12h, 4°C) with either IDD or control sera (25μl), or GAD-6 (Chang, *et al.*, *J.Neuro*, 8:2123-2130, 1988) (1μl in 25μl Tris buffer). Following another incubation with protein A Sepharose CL-4B (1h, 4°C), the complexes were then washed 5 times with 50mM Tris HCL (pH 7.4) with 0.1% SDS, 1.0% Triton X-114, and 2mM EDTA, and then washed again one time in double distilled water. The protein A Sepharose CL-4B was then boiled in Laemmli sample buffer (Laemmli, *Nature*, 227:680-685, 1970), and the samples were subjected to SDS-PAGE and fluororadiography (Kodak, X-omat AR5) using Enhance (New England Nuclear). Alternatively, the autoradiographs were analyzed by a BETAGEN (Boston, MA) analyzer. Both 64KA

positive and negative sera were used in each assay to serve as interassay controls. All fluororadiographs were analyzed and rated as positive or negative after comparison with the known interassay controls. Positive serum samples were designated as 1 when a sample resulted in immunoprecipitation of a low intensity 64,000 M_x band, 2 if a moderate intensity band was observed and 3 if the intensity of the immunoprecipitated protein was high. A similar rating procedure was employed for the intensity of bands corresponding to immunoprecipitated ^{35}S -GAD₆₅ and ^{35}S -GAD₆₇.

6. Immunoprecipitations. Immunoprecipitation of bacterial lysates containing ^{35}S -GAD₆₅ or ^{35}S -GAD₆₇, and GAD from human brain homogenate, was completed as described above in immunoprecipitation studies of human islet cell extractions.

7. GAD Assay. Human brain homogenates were incubated with patient sera as described above in human islet cells. After absorption and washes, the protein A agarose slurry was aliquoted into three equal volumes and GAD activity was measured as described (Krieger, *et al.*, *Neurochem.* 33:299, 1984). Briefly, Protein A agarose beads were incubated with (1- ^{14}C)-glutamate (Amersham) in a designated incubation mixture (Krieger, *et al.*, *Neurochem.* 33:299, 1984) and production of $^{14}\text{CO}_2$ was quantitated by a liquid scintillation counter.

8. Production of ^{35}S -GAD₆₅ and ^{35}S -GAD₆₇. Rat GAD₆₅ and GAD₆₇ cDNAs were subcloned into a bacterial expression system as previously described. Labeling of ^{35}S -GADs was completed by pulsing IPTG induced bacterium (growing in Minimal Media) for 15 minutes with TRAN ^{35}S -label (ICN). Cultures were then spun down and resuspended and sonicated in 1ml of homogenizing buffer (1mM phenyl-methylsulfonyl fluoride (PMSF), 1mM 2-aminoethylisothiuronium Bromide (AET) and 60mM potassium phosphate, pH 7.1). After sonication, cell debris was removed by centrifugation and protein concentration was measured (Bradford, *Anal.Biochem.*, 72:248, 1986) in the supernatant (supernatant was stored in aliquots at -70 °C).

B. IMMUNOREACTIVITY OF IDDM SPECIMENS

Sera from patients with IDDM were tested for the ability to precipitate GAD from human brain homogenates.

TABLE 4

SERA FROM IDDM PATIENTS IMMUNOPRECIPITATE GAD ACTIVITY

Patient	IDDM	Pre-IDDM Period ¹	64K ²	JDF ³	GAD Activity ⁴ SUM ⁵
DA	+	>24	3	164	13,762
DC	+	>1	3	20	1,719
HS	+	5	3	40	588
HL	+	0	2	80	440
HH	+	>1	2	10	184
C	-	na	0	0	280
C	-	na	0	0	285
C	-	na	0	0	325
C	-	na	0	0	275
C	-	na	0	0	270

¹ Expressed as months² 64K citors as described in Experimental Methods³ The islet cell antibody test as expressed in Juvenile Diabetes Foundation (JDF) units⁴ Not adjusted for background⁵ At risk for diabetes (also, failed glucose test)

na - Not applicable

As shown in Table 4, the sera of four (out of five) at risk for IDDM or IDDM patients bound significantly greater amounts of enzymatically active GAD of human brain extracts than sera from control patients. In addition, sera from one of the patients was drawn in a pre-IDDM period, thus autoantibodies to GAD are present prior to the onset of IDDM symptoms (see C below).

Further experiments (results not presented) showed that the sera of two at risk IDDM patients (DA, DC) immunoprecipitated recombinantly produced ³⁵S-GAD₆₅ whereas recombinantly produced ³⁵S-GAD₆₇ was only recognized by sera of patient DA (and to a lesser degree than ³⁵S-GAD₆₅). Subsequent studies have

found larger titers of GAD₆₇ autoantibodies than GAD₆₅ are present in sera of IDDM patients with neuropathic complications (not shown here).

Additional studies using patient DA sera showed the presence of antibodies which recognize specific polypeptides produced in human pancreatic islet cells. Electrophoretic analysis of the bound polypeptides demonstrated the presence of autoantibodies to a 64kD component, as previously shown by others in human IDDM (Baekkeskov, *et al.*, *Nature*, 298:167-169, 1982) and in animal models (Baekkeskov, *et al.*, *Science*, 224:1348-1350, 1984; Atkinson, *et al.*, *Diabetes*, 37:1587-1590, 1988). Prior absorption of these sera with GAD-6 monoclonal, which recognized GAD₆₅ but not GAD₆₇, or with bacterially produced GAD₆₅, abolished the ability of the sera to recognize the 64kD pancreatic polypeptide. The epitopes recognized by autoantibodies to the 64kD autoantigen are thus present in GAD₆₅, indicating that the autoantigen is indeed GAD₆₅. In order to investigate the predictive value of GAD₆₅, sera drawn from patients prior to onset of clinical manifestation of IDDM were tested for autoantibodies to GAD₆₅.

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TABLE 5

IDDM PATIENTS ANALYZED FOR AUTOANTIBODIES PRIOR TO THE ONSET OF DISEASE

Patient	Sex	HLA	Age Onset ¹	Pre-IDDM Period ²	JDF	64kD ³	GAD ₆₅ ⁴	GAD ₆₇ ⁴
JA	M	3,2	17	11	20	2	0	1
CA	F	4,5	38	4	0	1	1	0
RA	M	2,1	5	34	0	2	1	0
TB	M	2,4	11	66	40	1	1	0
AB	M	N.D.	23	6	160	3	3	2
VC	F	4,6	15	3	40	1	0	1
JD	M	6,1	34	25	10	3	1	1
DR	F	3,4	14	42	320	2	1	0
JG	M	3,3	12	8	40	1	0	0
BR	M	3,3	5	9	0	0	1	1
KR	F	4,X	34	14	10	3	2	0
JT	F	4,6	7	10	N.D.	1	1	1

¹ Age of IDDM onset expressed as months² The time interval between sera drawn and IDDM onset expressed as months³ 1 - lowest; 2 - medium; and 3 - highest band intensities

N.D., not determined

As shown in Table 5, 9 out of 12 specimens (75%) were immunoreactive with ³⁵S-GAD₆₅. In addition, two patients (JA and VC) were immunoreactive to GAD₆₇, but not GAD₆₅ under these conditions. Therefore, in combination, autoantibodies to GAD₆₅ and GAD₆₇ were present in 11 out of 12 (91%) of these patients sera. This finding suggests that although autoantibodies to GAD₆₅ are more common than autoantibodies to GAD₆₇, the use of both recombinant GADs (GAD₆₅ and GAD₆₇) in an assay would allow for greater predictability of IDDM. Previous tests of these sera (Atkinson, *et al.*, *Lancet*, 335:1357-1360, 1990) demonstrated that 11 out of 12, or 92%, immunoreacted with the ³⁵S-64kD molecule from human pancreatic islet cells. The serum which contained detectable autoantibodies to the 64kD molecule and not GAD₆₅ was a serum which contained the lowest titer (or "1") for the 64kD molecule. Thus, the false negative obtained

was due to a lack of sensitivity in this assay. Furthermore, this assay predicted IDDM in one patient (BR) who was negative for 64K.

These results show that the 64kD molecule identified in β -cells of human pancreas is identical in size and antigenicity to rat GAD₆₅. Furthermore, sera drawn from patients prior to IDDM onset contain autoantibodies to GAD₆₅. Consequently, the GAD₆₅ recombinant molecule is of great utility as a diagnostic tool for predicting IDDM. The ability of a physician to diagnose IDDM prior to actual symptoms will no doubt result in a greater extension of time before insulin therapy is needed. The sensitivity of such immunoassays will improve with the use of a recombinant GAD₆₅ of human origin which represents the GAD form present in β -cells of the pancreas.

EXAMPLE 4

IMMUNE PROLIFERATIVE RESPONSE TO POLYPEPTIDE

Polypeptides were synthesized using an automatic instrument (Applied Biosystems) and standard conditions. These polypeptides were then tested to compare their relative ability to stimulate proliferation of splenic lymphocytes and islet infiltrating T lymphocytes (IITLs). In this study, polypeptides derived from the GAD₆₅ core sequence and from the homologous region of polio virus were compared. Appropriate cells were cultured for 5 days with the respective polypeptide in the presence of 5×10^4 irradiated spleen cells. ³H-thymidine was added during the last 16 hours of culture.

TABLE 6

ANTIGEN	AMINO ACID SEQUENCE	³ H-THYMIDINE INCORPORATION (cpm) BY LYMPHOID CELL POPULATION	
		IITLs ^a	SPLEEN ^b
None	-	1,100	6,500
Poliovirus	MKSMCPQAQLKVKYL	900	22,500
GAD ₆₅	ARFKMFPEVKEKGMAA	9,500	23,300

^a islet infiltrating T lymphocytes (3×10^4 cells/well)

^b 1×10^5 cells/well

In these studies, there was no significant difference in the proliferative activity of cultures of spleen lymphocytes exposed to either the polio or the GAD₆₅ polypeptides. However, both polypeptides stimulated a T cell response which was higher than that found in the media control. The lack of difference in proliferation in the spleen cell population may be due to a lower frequency of GAD polypeptide specific T cells.

The IITL population, when evaluated in the same manner, showed a marked difference in cell proliferation. In this system, the response to the GAD₆₅ polypeptide was 9-fold greater than that of either the culture media or the polio polypeptide. This data strongly suggests that the GAD₆₅ is an important antigen for T cell responses in the IITL population. This data suggests that molecular mimicry plays a role in the pathogenesis of diabetes.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made without departing from the spirit or scope of the invention.

Claims

1. An isolated polypeptide or analog, chemical derivative, or pharmaceutically acceptable salt thereof having the following amino acid sequence:

X - Pr - Glu - Val - Lys - Y - Lys - Z

wherein X is an amino acid sequence selected from one to ten amino acids or omitted; Y is Thr or Glu; and Z is an amino acid sequence selected from one to eight amino acids or omitted.

2. The polypeptide of claim 1, wherein X comprises Lys, Y is Thr and Z comprises Leu.

3. The polypeptide of claim 1, wherein X comprises Lys, Y is Glu, and Z comprises Leu.
4. The polypeptide of claim 3, wherein X further comprises Met and Z further comprises Arg and Leu.
5. The polypeptide of any one of claims 1 to 3, wherein X is the amino acid sequence Ser - Ile - Met - Ala - Ala - Arg - Tyr - Lys - Tyr - Phe - and Z is the amino acid sequence Gly - Met - Ala - Ala - Val - Pro - Lys - Leu.
6. The polypeptide of any one of claims 1 to 4, wherein X is the amino acid sequence Ala - Met - Met - Ile - Ala - Arg - Phe - Lys - Met - Phe and Z is the amino acid sequence Gly - Met - Ala - Leu - Pro - Arg - Leu.
7. An isolated polynucleotide sequence encoding the polypeptide of any one of claims 1 to 6.
8. The polynucleotide of claim 7 which is DNA.
9. The polynucleotide of claim 8 which is cDNA.
10. An antibody to the polypeptide of any one of claims 1 to 6.
11. The antibody of claim 10, which is a monoclonal antibody.
12. A method of providing the polypeptide of any of claims 1 to 6, comprising:
 - (a) providing a host cell transformed with a polynucleotide of any one of claims 7 to 9;
 - (b) growing the host cell; and
 - (c) recovering the polypeptide.
13. A pharmaceutical composition comprising a therapeutically effective amount of the polypeptide of any one of claims 1 to 6.
14. Use of the polypeptide of any one of claims 1 to 6 for the preparation of a medicament for the treatment of autoimmune diseases.
15. A diagnostic kit comprising the polypeptide of any one of claims 1 to 6 and/or the antibody of claim 11 or 12.

feline (from cDNA)	<u>YEIAPVFVLM</u> EQITLKKREIVGWSSKDGDI F SPGAISNMYSIMAA R YK F <u>FEVKT</u> KG
	→
rat (from peptide)	<u>YEIAPVFVL</u> LEYV-----REIIGWPGGS-DG I F SPGAISN-YAM L A R YK M <u>FEVKE</u> KG

FIG. 1

FIG. 2A

```

GGCGTGGGGGTGAGCCGAGCAGCTTGCCCCGAGCCACTCGAGGCGAGCCGCCA
10
      M A S P G S G F W S F G S E D G
GACTAGCAGAACCCATGGCATCTCCGGGCTCTGGCTTTGGTCCTTCGGATCTGAAGATG
70
      S G D P E N P G T A R A W C Q V A Q K F
GCTCTGGGATCCTGAGAACCCGGGAACAGCGAGAGCCTGTGCCAGGTGGCCCAAGT
130
      T G G I G N K L C A L L Y G D S E K P A
TCACGGCGGCATCGGAACAAGCTATGCGCTCTGCTCTACGGAGACTCTGAGAACCCAG
190
      E S G G S V T S R A A T R K V A C T C D
CAGAGAGCGCGGAGCGTGACCTCGCGGCGCCCACTCGGAAGTCCGCTGCACCTGTG
250
      Q K P C S C P K G D V N Y A L L H A T D
ACCAAAACCCTGCAGCTGCCCAAGAGATGCAATTATGCACCTCTCCACGCAACAG
310
      L L P A C E G E R P T L A F L Q D V M N
ACCTGCTGCCAGCCTGTGAAGGAAAGGCCCACTCTCGCATTTCTGCAAGATGTAATGA
370
      I L L Q Y V V K S F D R S T K V I D F H
ACATTTGCTTCAGTACGTGTGAAGAAAGTTTGATAGATCAACTAAGTGTGATTTCC
430
      Y P N E L L Q E Y N W E L A D Q P Q N L
ATTACCCCAATGAGCTTCTTCAAGAGTATAATTGGGAATTGGCAGACCAACGCAAAATC
490
      E E I L T H C Q T T L K Y A I K T G H P
TGGAGGAAATTTGACCGCACTGCCAAACAACCTCTAAATATGCGATTAAACAGGGCATC
550
      R Y F N Q L S T G L D M V G L A A D W L
CCCGATATTTAATCAGCTGTCTACCGGATTTGATATGTTGATTAAGCAGCATTTGGT
610
      T S T A N T N M F T Y E I A P V F V L L
TGACATCAACAGCAACACGAACATGTTTACCTATGAGATCGCCCTGTATTGTACTAC
650

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FIG. 2B

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E Y V T L K K M R E I I G W P G G S G D 670 690 710
TGAATATGTGACACTAAAGAAAATGAGGAATCATTTGGCTGGCCAGGAGGCTCTGGCG
730
G I F S P G G A I S N M Y A M L I A R Y 750 770
ATGAATCTTTTCTCTCCCTGGTGCGCATCTCCAACATGTACGCCATGCTCATTGCCGCT
790
K M F P E V K E K G M A A V P R L I A F 810 830
ATAAGATGTTCCAGAGTCAGGAAGGAGGATGCCGCGGTGCCAGGCTCATTCGCAT
850 870 890
T S E H S H F S L K K G A A A L G I G T
TCACGTCAGAGCATAGTCACTTTTCTCTCAAGAGGAGCTGCAGCCTTGGGATCGAA
910 930 950
D S V I L I K C D E R G K M I P S D L E
CAGACAGCGTGATTTCTGATTAATGTGATGAGAGGAGGAAATGATCCCATCTGACCTTG
970 990 1010
R R I L E V K Q K G F V P F L V S A T A
AAAGAAGAATCCTTGAAGTCAACAGAAAGGATTTGTTCTTCTTCTGAGTGCCACAG
R R I L E V K Q K G F V P F L V S A T A
AAAGAAGAATCCTTGAAGTCAACAGAAAGGATTTGTTCTTCTTCTGAGTGCCACAG
1030 1050 1070
G T V Y G A F D P L L A V A D I C K K
CTGAACCACTGTGTACGGGCTTTTGATCCTCTCTTGGCTGTAGCTGACATCTGCAAA
1090 1110 1130
Y K I W M H V D A A W G G G L L M S R K
AATATAAGATCTGATGTCATGTGATGCTGCTTGGGGTGAGGGTTACTGATGTCTCGA
1150 1170 1190
H K W K L N G V E R A N S V T W N P H K
AACACAAGTGAAGCTGAACGGGTGTGAGAGGGCCAACTCTGTGACATGGAATCCCCACA
1210 1230 1250
M M G V P L Q C S A L L V R E E G L M Q
AGATGATGGGTGTCCTTGCATGTTCGGCTCTCTGCTGTCAGAGAGGAGGACTGATGC

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FIG. 2C

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1270      S C N Q M H A S Y L F Q Q D K H Y D L S
AGAGCTGCAACCAGATGCATGCTTCCCTACCTCTTTCAGCAAGATAAGCACTATGACCTGT
1330      Y D T G D K A L Q C G R H V D V F K L W
CCTATGACACGGGAGACAAAGGCCCTTGCAGTGTGGACGCCACGTCGATGCTCTTAAATTAT
1390      L M W R A K G T T G F E A H I D K C L E
GGCTCATGTGAGAGCAAGGGGACTACTGGATTGGAAGCTCACATTGATAAGTGTGG
1450      L A E Y L Y N I I K N R E G Y E M V F D
AGCTGCAGAGTATTATTACAATATCATTAATAAACCGAAGGATATGAATGGTTCG
1510      G K P Q H T N V C F W F V P P S L R V L
ATGGGAGCCCTCAGCACACAATGCTGCTCTGCTTGTGTACCTCCTAGTTGCGAGTTC
1570      E D N E E R M S R L S K V A P V I K A R
TGGAAGACAATGAAGAGAGAATGAGCCGCTCTCAAGGTGGCCGAGTGAATTAAGCCA
1630      M M E Y G T T M V S Y Q P L G D K V N F
GAATGATGAGTATGGACCACAATGTCAGCTACCAACCTTAGGAGATAAGTCACT
1690      F R M V I S N P A A T H Q D I D F L I E
TCTTCCGCATGGTCATCTCAAAACCTGCAAGCACTCAACCAAGACATTGACTTCCTCATTG
1750      E I E R L G Q D L *
AAGAAATCGAACGCCCTGGACAAAGATTGTATACTTTGCTCACCAAACTTTCAGTTCT
1810      CTAGGTAGACAGCTAAGTTGTCACAAACCTGTGTAATGTATTGTGTTGCCAGAGT
1870      AATTCTATTCTATATATCGTGCTGTCAACAGTAGAGTCCAGTTTAAAA
1930

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FIG. 3A

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agctcgcccgagctcgcaactcgagggcgacctgtctccagttctccaagccgatggcattc
10
P G S G F W S F G S E D G S G D S E N P
tcgggtcttggtcttttggtcttccgggtcggagatggctctgggattccggaattcc
70
G T A R A W C Q V A Q K F T G G I G N K
cggcacagcgcgagagctgtgcacagtggtcagaagttcacgggctggcatcggaacaa
130
L C A L L Y G D A E K P A E S G G S Q P
actgtgcgcctgtcttaacggagagcgccggcgagagcgcgaggagccaacc
190
P R A A A R K A A C A C D Q K P C S C S
cccgcgggcccgcccggaagcgccgtgctgcgcctgcgaccagaagccctgcagctgtc
250
K V D V N Y A F L H A T D L L P A C D G
caaagtggatgtcaactacgcgttcttccatgcacaacagacctgtgcggcggtgtgatgg
310
E R P T L A F L Q D V M N I L L Q Y V V
agaaaagcccaacttggcggttcttgcgaagatgttatgaacatttaccctcagttatgtgt
370
K S F D R S T K V I D F H Y P N E L L Q
gaaaagtcttcgatatgatcaaaccaagtgattgttccattatcccaatgagcttccca
430
E Y N W E L A D Q P Q N L E E I L M H C
agaatataattgggaattggcagagaccaacaacaatttggaggaatatttgatgcatgg
490
Q T T L K Y A I K T G H P R Y F N Q L S
ccaaacaactctaataatgtcaattaaaacaggggcatcctagatacttcaatcaacttcc
550
T G L D M V G L A A D W L T S T A N T N
tactggtttggatatggttggtttagcagcagagctggctgacatcaacagcaataactaa

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M F T Y E I A P V F V L L E Y V T L K K 610 630 650
 catgtcacctatgaaattgtccagtatctgtgtcttttggaatatgtcacactaaagaa 670 690 710
 M R E I I G W P G G S G D G I F S P G G 730 750 770
 aatgagagaaatcattggctggccagggggctctgtggcatgggatatcttctcccggtg
 A I S N M Y A M M I A R F K M F P E V K 790 810 830
 cggcatatctaacaatgtatgccatgatgcgcacgctttaagatgttccagaagtcaa
 E K G M A A L P R L I A F T S E H S H F 850 870 890
 ggaagaaggaaatgctgtctctcccaagctcattgtcctcacgtctgaacatagtcattt
 S L K K G A A A L G I G T D S V I L I K 910 930 950
 ttctccaagaaggagctgcagccttagggattggaaccagacagcgtagtcttgattaa
 C D E R G K M I P S D L E R R I L E A K 970 990 1010
 atgtgatgagagagggaaatgatccatctgatcttgaagaaggattcttgaagccaa
 Q K G F V P F L V S A T A G T T V Y G A 1030 1050 1070
 acagaaagggttctgtcccttccctcgtgagtgccacagctggaaccacggtgacgagc
 F D P L L A A V A D I C K K Y K I W M H V 1090 1110 1130
 atttgacccccctcttagctgtcgtgcacatttgcaaaaagtataagatctggatgcargt
 D A A W G G G L L M S R K H K W K L S G 1150 1170 1190
 ggatgcagcttgggtgggggattactgatgtcccgaaaacacacaagtggaaactgagtgg
 V E R A N S V T W N P H K M M G V P L Q
 cgtggaagaggccaactctgtgacgtggaatccacacaagatgatgggagtccttgc

FIG. 3B

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C S A L L V R E E G L M Q N C N Q M H A 1210 1230 1250
gtgctctgctcctcctgtgtagaagaagggttgatgcaagaattgcaaccaaatgcatgc
S Y L F Q Q D K H Y D L S Y D T G D K A 1270 1290 1310
ctcctaccctcttcagcaagataaacattatgaccctgtcctatgaccactggaagacaagc
L Q C G R H V D V F K L W L M W R A K G 1330 1350 1370
ctaactgtgcggaccgcaacgttgatgtttttaactatggctgatgtggagggaaggg
T T G F E A H V D K C L E L A E Y L Y N 1390 1410 1430
gactaccgggtttgaaagcgcattgttgataaatgtttggagttggcagagtattatacaa
I I K N R E G Y E M V F D G K P Q H T N 1450 1470 1490
catcataaaaaaccgaagaagatatgagatgtgtttgatgggaagcctcagcacaacaaa

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FIG. 3C

1510 1530 1550
V C F W Y I P P S L R T L E D N E E R M
tgcctgtctgtgtacattccctccaagcttgctgactctggaagacaatggaagagaat
1570 1590 1610
S R L S K V A P V I K A R M M E Y G T T
gagtcgacctctcgaaagtgtgctccagtgtattaaagccagaatgatggaatggaaccac
1630 1650 1670
M V S Y Q P L G D K V N F F R M V I S N
aatgtcagctaccacaaccttgaggagacaaggtcaatttctccgcattgtcattctcaa
1690 1710 1730
P A A T H Q D I D F L I E E I E R L G Q
ccaagcggaactcaccagaacattgacttccctgattgaaagaatagaacgaccttgaca
1750 1770
D L *
agatttataataacccttgctcaccagaagctgttccacttctctaagtagacaattaaattg
1810 1830 1850
tcacaaactgtgtgaatgtattgtagtttgttccaaagtaaatctatttctatatgtg
1870 1890 1910
gtgtcaaaagttagatttaaaaattaaacaaaagacattgctcctttaaagtccttt
1930 1950 1970
cttaagtttaagaatacccttctaagaattcgtgacaaaaggtatgttctaataatgaag
1990 2010 2030
gaaaagcttaaaattgttataataacttcccttacttttaatatagtggtgcaagcaaac
2050 2070 2090

FIG. 3D

GAP WEIGHT:	3.000	AVERAGE MATCH:	0.540
LENGTH WEIGHT:	0.100	AVERAGE MISMATCH:	-0.396
QUALITY:	856.2	LENGTH:	585
RATIO:	1.464	GAPS:	0
PERCENT SIMILARITY:	97.436	PERCENT IDENTITY:	96.068

4817, PEP HGT2, PEP

AUGUST 22, 1990 08:20 ..

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1  MASPGSGFWFSGSEDGSGDPENPGTARAWCQVAQKFTGGIGNKLCALLYG  50
1  MASPGSGFWFSGSEDGSGDSENPGTARAWCQVAQKFTGGIGNKLCALLYG  50
51  DSEKPAESGGSVTSRAATRKVACTCDQKPCSCPKGDVNYALLHATDLLPA  100
51  DAEKPAESGGSQPPRAAARKAACACDQKPCSCSKVDVNYAFLHATDLLPA  100
101  CEGERPTLAFLQDVMNILLQYVVKSFDRSTKVIDFHYPNELLQEYNWELA  150
101  CDGERPTLAFLQDVMNILLQYVVKSFDRSTKVIDFHYPNELLQEYNWELA  150
151  DQPQNLEEILTHCQTTLKYAIAKTGHPRYFNQLSTGLDMVGLAADWLTSTA  200
151  DQPQNLEEILMHCQTTLKYAIAKTGHPRYFNQLSTGLDMVGLAADWLTSTA  200
201  NTNMFITYEIAPVFVLLLEYVTLKKMREIIGWPGGSGDGI FSPGGAISNMYA  250
201  NTNMFITYEIAPVFVLLLEYVTLKKMREIIGWPGGSGDGI FSPGGAISNMYA  250
251  MLIARYKMFPEVKEKGMAAVPRLIAFTSEHSFSLKKGAAALGIGTDSVI  300
251  MMIARFKMFPEVKEKGMAALPRLIAFTSEHSFSLKKGAAALGIGTDSVI  300
301  LIKCDERGMIPSDLERRILEVKQKGFVPFLVSATAGTTVYGAFDPLLAV  350
301  LIKCDERGMIPSDLERRILEAKQKGFVPFLVSATAGTTVYGAFDPLLAV  350
351  ADICKKYKIWMHYDAAWGGGLLSRKHKVKLNGVERANSVTWNPHKMMGV  400
351  ADICKKYKIWMHYDAAWGGGLLSRKHKVKLSGVERANSVTWNPHKMMGV  400

```

FIG. 4A

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401 PLQCSALLVREEGLMQSCNQMHASYLFQQDKHYDLSYDTGDKALQCGRHV 450
   |||||||||||||||.|||||||||||||||||
401 PLQCSALLVREEGLMQNCNQMHASYLFQQDKHYDLSYDTGDKALQCGRHV 450
   .      .      .      .      .
451 DVFKLWLMWRAKGTGFEAHIDKCLELAEYLYNI IKNREGYEMVFDGKPO 500
   |||||||||||||||:|||||||||||||||||
451 DVFKLWLMWRAKGTGFEAHVDKCLELAEYLYNI IKNREGYEMVFDGKPO 500
   .      .      .      .      .
501 HTNVCFWFVPPSLRVLEDNEERMSRLSKVAPVIKARMMEYGTTMVSQPL 550
   |||||:|:|||||.|||||||||||||||||
501 HTNVCFWYIPPSLRTLEDNEERMSRLSKVAPVIKARMMEYGTTMVSQPL 550
   .      .      .      .      .
551 GDKVNFFRMVISNPAATHQDIDFLIEEIERLGQDL 585
   |||||||||||||||
551 GDKVNFFRMVISNPAATHQDIDFLIEEIERLGQDL 585

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FIG.4B

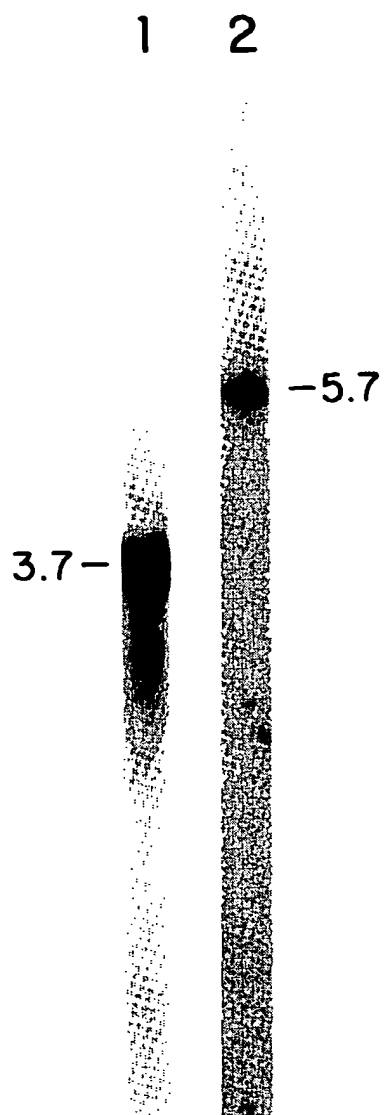


FIG. 5

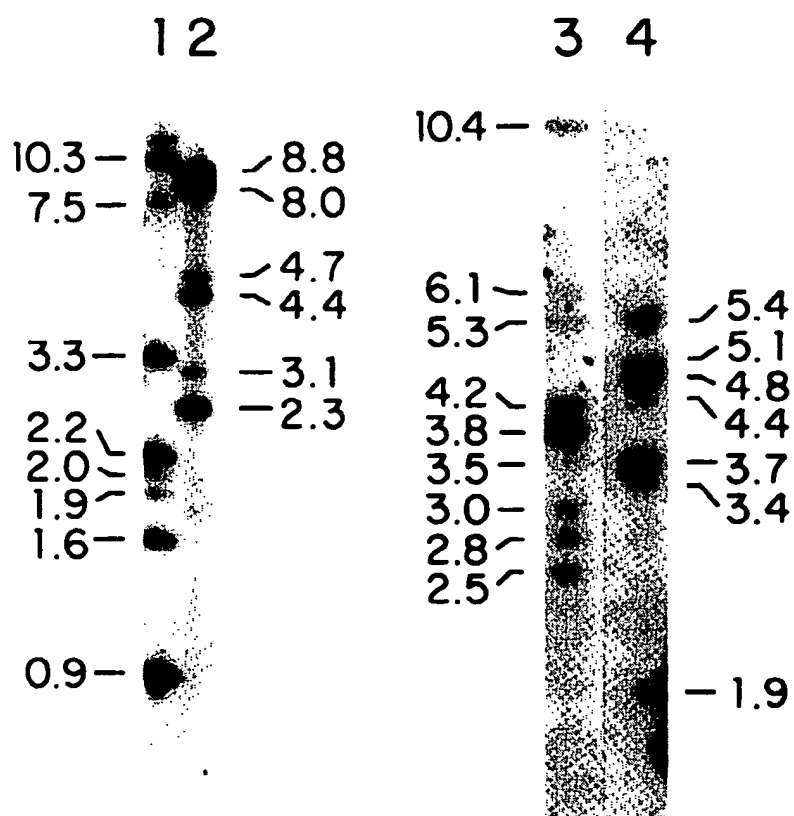


FIG. 6

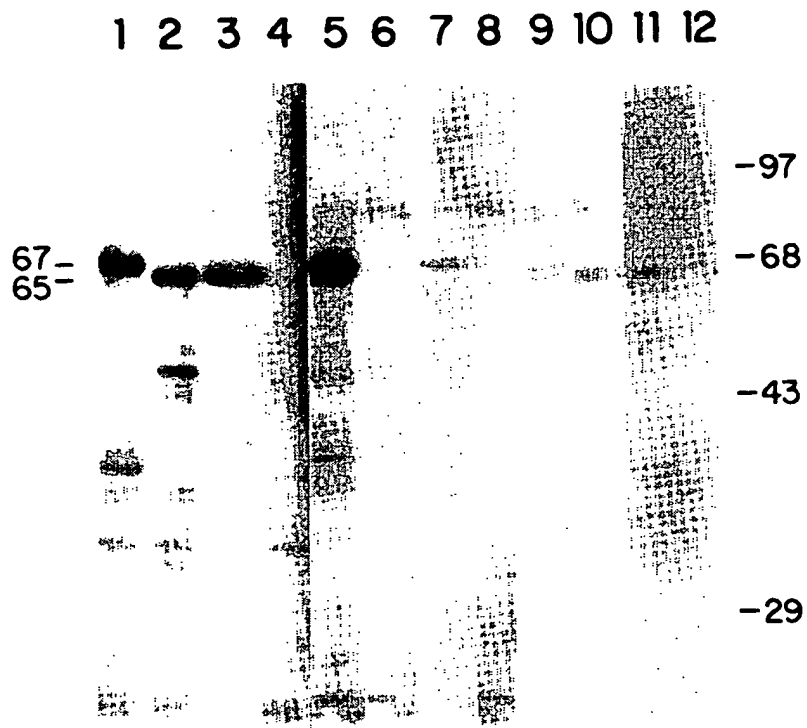


FIG. 7



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Application Number

EP 92110308.1

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Application Number

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			TECHNICAL FIELDS SEARCHED (Int. Cl.5)
The present search report has been drawn up for all claims			
VIENNA		Date of completion of the search 24-09-1992	Examiner SCHARF
CATEGORY OF CITED DOCUMENTS X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background : non-written disclosure P : intermediate document T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document			